JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Time Course Study of Substrate Utilization by *Aspergillus flavus* in Medium Simulating Corn (*Zea mays*) Kernels

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Utilization of the three major corn reserve materials, starch, triglycerides (refined corn oil), and zein (storage protein), by *Aspergillus flavus* was monitored in vitro over a 7-day fermentation. Medium composition in which proportions of reserve materials initially approximated proportions in mature corn kernels changed little over the first 18 h. Subsequently, hydrolysis of both starch and triglycerides occurred simultaneously, with peak concentrations of glucose and free fatty acids on day 2 of the fermentation period. Fatty acid concentrations dropped relatively rapidly after day 2 but increased again after day 6. Aflatoxin B_1 production increased after 36 h, with a peak at day 4. Aflatoxin B_1 production paralleled fungal biomass production during the exponential growth phase. *A. flavus* did not appear to preferentially utilize any of the released fatty acids. A number of fungus-specific metabolites were detected, including arabitol, erythritol, mannitol, trehalose, and kojic acid. Mannitol exceeded the other metabolites in concentration, and the timing of mannitol production closely paralleled that of aflatoxin B_1. Kojic acid concentrations peaked at day 6. In contrast to previously described selective use of simple carbohydrates by *A. flavus*, less discrimination was displayed when faced with utilization of complex substrates such as starch or triglycerides.

KEYWORDS: Aflatoxin; *Aspergillus flavus*; corn lipids; cornstarch; *Zea mays*; zein

INTRODUCTION

Aspergillus flavus is a widely distributed saprophytic fungus that is pathogenic to oilseed crops (cotton, peanuts, and tree nuts) and corn (*Zea mays*) under certain environmental conditions. The fungus can produce the potent carcinogen aflatoxin during seed infection, resulting in diminished crop values.

Reserve materials of corn include starch, lipids, and zein, a member of the prolamin class of storage proteins. Corn reserve materials are localized in the endosperm and seed germ. Corn contains 75% starch and 5% lipids (triglycerides) by weight and, in addition, 11% protein, with zein contributing 50% of the total protein component (*1*). Corn kernel reserves represent significant carbon and nitrogen resources potentially available during seed infection by *A. flavus*.

Oilseed storage proteins are known to stimulate aflatoxin production by *A. flavus*. The presence of zein as a supplement in chemically defined culture media stimulates aflatoxin production up to 10-fold, and its presence as a sole nitrogen source together with a rapidly accessible carbon source stimulates aflatoxin production by 8-fold over chemically defined medium reference cultures (*2*). Similarly, the presence of raffinose as a carbon source and cottonseed storage protein as a sole nitrogen source stimulates aflatoxin production 4-fold over chemically defined growth media (*3*). Furthermore, in a study utilizing media that contained the major reserve materials in ratios similar to those found in mature cottonseed, *A. flavus* selectively used raffinose for initial biomass and aflatoxin production (*4*). Thus, the fungus appears to require a rapidly accessible saccharide to initiate rapid aflatoxin production. A hexose transport gene has been found associated with the aflatoxin biosynthesis gene cluster (*5*).

To further investigate the utilization of corn reserve materials by *A. flavus* during aflatoxin biosynthesis, a defined culture medium incorporating the major storage components of corn was employed. The medium contained cornstarch, lipids (triglycerides), and zein in ratios similar to those in planta (seed) and was used to assess the sequence of substrate utilization with respect to biosynthesis of aflatoxin and other fungus-specific metabolites.

MATERIALS AND METHODS

Biological Materials. *Aspergillus flavus* AF13 (ATCC 96044) was isolated from soil in southwestern Arizona (Yuma area) and maintained on a 5% V-8 vegetable juice (Campbell Soup Co., Camden, NJ) agar medium at 31 °C (*6*). Culture medium was seeded (200 μL/70 mL) with a conidial suspension containing 10^6-10^7 spores/mL. Zein was purchased from Sigma Chemical Co. (St. Louis, MO). Refined corn oil was produced by Lou Ana Foods, Inc. (Opelousas, LA). Refined cornstarch was produced by Best Foods (Englewood Cliffs, NJ).

Fungal Incubations. A chemically defined culture medium (*7*) was used throughout this study without the standard carbon and nitrogen sources. This medium was supplemented with cornstarch (2.45 g/70

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10.1021/jf011048e
Published on Web 12/28/2001

mL), corn oil (0.53 g/70 mL), and zein (0.53 g/70 mL) in proportions (14:3:3, respectively) similar to those in mature corn kernels on a dry weight basis (*1*). Incubations were conducted in 250-mL flasks (70 mL/flask); media were adjusted to pH 5.0 before sterilization (autoclaving). Reference control cultures contained glucose (3.5 g/70 mL) as a carbon source and $NaNO_3$ (0.21 g/70 mL) as a nitrogen source. Fermentations were performed on a rotary shaker at 31 °C and 200 rpm in the dark. Fermentations were monitored from 0 to 7 days for pH and concentrations of total reducing sugar, aflatoxin B_1, saccharides, sugar alcohols, fatty acids, starch, zein, and biomass.

Reducing Sugar Assay. After each culture was removed from the shaker, the pH was measured and a 1-mL aliquot of growth medium was removed. Aflatoxin was removed by passage through a reversed-phase C_{18} cartridge (*4*), and reducing sugar concentrations were determined according to the *p*-hydroxybenzoic acid hydrazide method (*8*). Glucose was used as a standard.

Aflatoxin Analysis. Following removal of the reducing sugar sample, cultures were terminated and aflatoxins solubilized with 33% (v/v) acetone. The resulting solution was spotted directly beside aflatoxin standards (B_1, B_2, G_1, and G_2) on silica gel G plates. After development in diethyl ether/methanol/water (96:3:1), aflatoxin B_1 was quantified directly on thin layer plates by fluorescence densitometry (*9*) with a model CS-9301 PC densitometer (Shimadzu, Kyoto, Japan).

Lipid/Saccharide Analysis. Following aflatoxin analysis, the liquid portion of each culture was removed by filtration in vacuo. The solid residue was washed with 25 mL of ethyl acetate to remove remaining lipids. This ethyl acetate wash fraction was pooled with the culture filtrate, and the mixture was vigorously swirled (~1 min) to emulsify the contents. A 2-mL sample of the emulsion was added to a Reacti-Vial (Pierce Chemical Co., Rockford, IL); acetone/ethyl acetate was removed by purging the emulsion with a stream of nitrogen. Samples were stored at −20 °C until lyophilized to remove water. Trimethylsilyl derivatives were produced, and samples were analyzed by gas chromatography as previously described (*4*). Quantitative analysis was performed by internal standardization. Experiments were conducted twice with three replicates. The results reported herein are representative of those experiments.

Metabolite concentrations reported in this work constitute a summation of intra- and extracellular levels due to lysing of fungal membranes with acetone prior to aflatoxin analysis. However, reducing sugar concentrations reflect extracellular levels because these samples were taken before membrane lysis.

Determination of Zein, Starch, and Fungal Biomass. Following lipid/saccharide sampling, the remaining residue from each culture was mixed with 100 mL of 60% (v/v) aqueous 2-propanol. The mixture was heated at 65 °C for ~30 min, shaken for 15 min, and reheated at 65 °C for 15 min. Some samples required several (two or three) cycles of this treatment to completely solubilize the remaining zein. After zein solubilization, the liquid fraction was separated from the solid residue by filtration in vacuo. 2-Propanol and water were removed from each sample by rotary evaporation. Each recovered zein sample was washed with 10 mL of methanol/chloroform (1:1) to remove traces of lipids, dried at 50 °C for 24 h, and weighed.

The remaining solid residue from each culture was added to 100 mL of deionized water, heated to boiling for 5 min, and filtered through MiraCloth to separate the starch fraction from biomass material (mycelia). Water was removed by rotary evaporation; each remaining starch sample was dried at 50 °C for 24−48 h and weighed. Remaining biomass material was dried at 50 °C and weighed.

RESULTS

The metabolic versatility of *A. flavus* was demonstrated during growth on the corn kernel simulation medium. Aflatoxin biosynthesis began after 24 h of fermentation with the greatest rate of production occurring between days 2 and 4. After aflatoxin production peaked (day 4), concentrations decreased over the next 3 days (**Figure 1**). Culture pH remained constant for the first day, followed by a large decrease until day 3; medium pH gradually increased during the remainder of the fermentation period (**Figure 1**). Fungal biomass increased significantly in a linear manner from 36 h until day 4, followed by a more gradual increase throughout the remainder of the fermentation period (**Figure 1**).

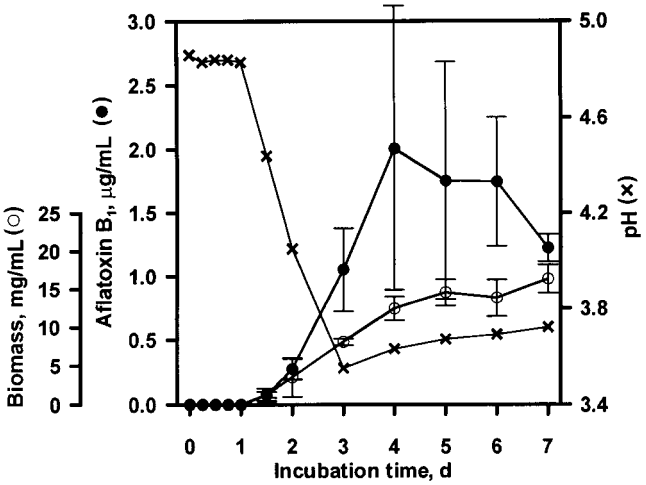

Figure 1. Effect of culture age on aflatoxin B_1 production, biomass production, and culture pH in a medium containing starch, triglycerides, and zein. Mean aflatoxin B_1 concentration ($n = 3$) is expressed in μg/mL of culture medium. Mean biomass production ($n = 3$) is expressed in mg of dry wt/mL of culture medium. Error bars represent standard deviations ($n - 1$).

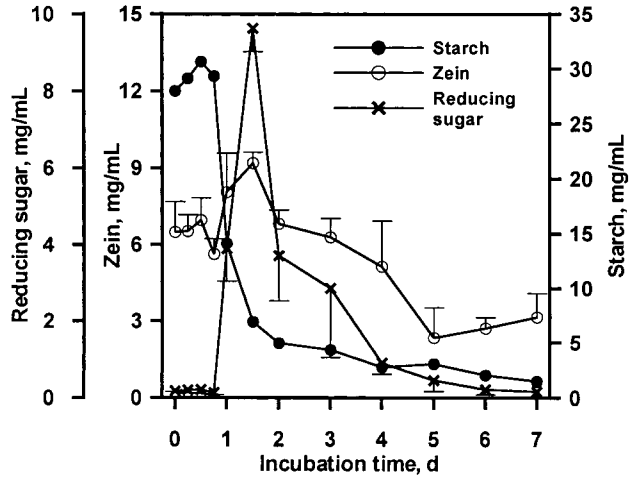

Figure 2. Substrate concentration profiles of *A. flavus* cultures grown 7 days in a medium containing starch, triglycerides, and zein. Concentrations are expressed in mg/mL of culture medium ($n = 3$). Error bars are not shown for starch data for clarity of presentation. Standard errors for starch data ranged from 0.01 to 0.35 mg/mL.

Carbohydrate concentrations changed significantly during the fungal fermentation period. Starch levels remained stable for 18 h and then dropped precipitously until 36 h, followed by a gradual decrease over the remainder of the fermentation period (**Figure 2**). The reducing sugar concentration greatly increased after 18 h to a peak at 36 h, followed by a large initial decrease that continued more gradually after 2 days (**Figure 2**). Glucose concentrations rose very sharply after 36 h to a peak at 2 days, followed by a continual decrease over the rest of the incubation period (**Figure 3**). Concentrations of maltose and isomaltose (α-1,6) mimicked those of glucose, albeit at considerably lower levels (**Figure 3**). Maltotriose, panose (α-1,6, α-1,4), maltotetraose, and maltopentaose concentrations also peaked at 2 days, but, relative to glucose, the concentrations of these components remained low throughout the fermentation period (data not

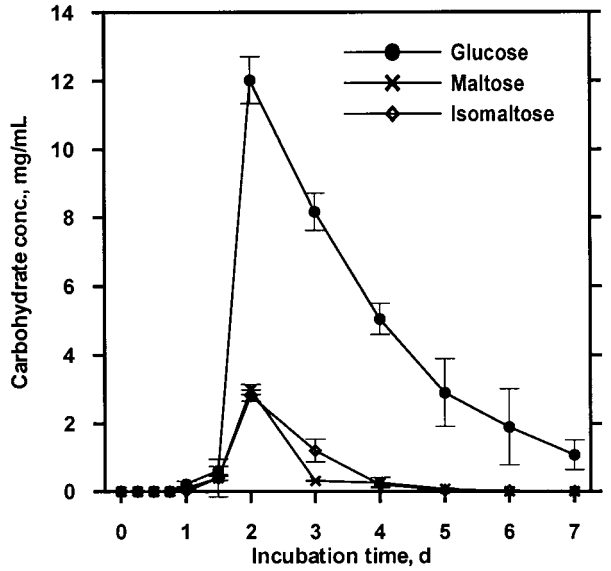

Figure 3. Carbohydrate concentration profiles of *A. flavus* cultures grown for 7 days in a medium containing starch, triglycerides, and zein. Concentrations are expressed in mg/mL of culture medium ($n = 3$).

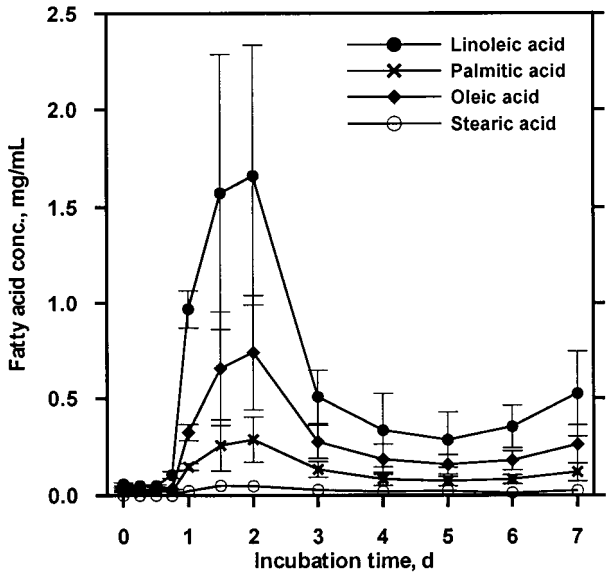

Figure 4. Fatty acid concentration profiles of *A. flavus* cultures grown for 7 days in a medium containing starch, triglycerides, and zein. Concentrations are expressed in mg/mL of culture medium ($n = 3$). Error bars are not shown for stearic acid data for clarity of presentation.

shown). Zein concentrations changed little until day 3 and then began a gradual decline (**Figure 2**). At 7 days, zein concentrations were approximately half of initial concentrations.

Free fatty acid concentrations remained low until after 18 h with a peak at 2 days, followed by a significant decrease (**Figure 4**). Fatty acid concentrations began to increase again at the end of the fermentation period (7 days). The distribution of individual fatty acids (linoleic, oleic, palmitic, stearic acids) was very similar to that of corn triglycerides. The concentration profiles were identical for all of the fatty acids released during triglyceride hydrolysis (**Figure 4**).

Levels of fungus-specific metabolites differed considerably, depending on the substance in question. Arabitol and erythritol (sugar alcohols) and trehalose concentrations gradually increased after 2 days but never exceeded 0.5 mg/mL (**Figure 5**). Mannitol concentrations rose after 36 h to a peak (3.8 mg/mL) at 4 days,

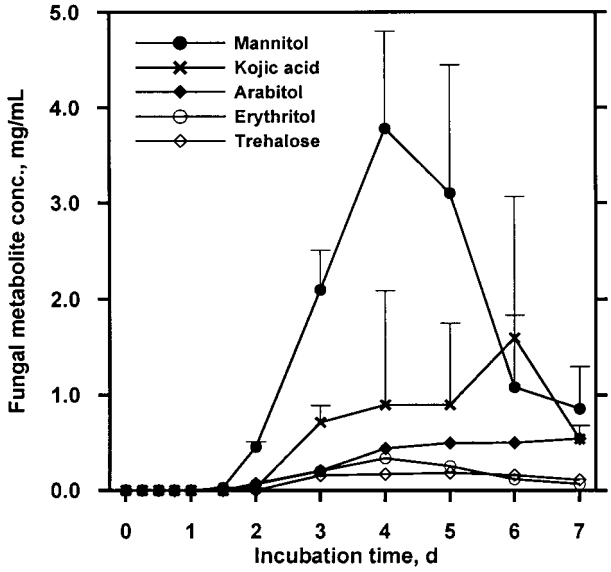

Figure 5. Concentration profiles of fungus-specific metabolites produced in *A. flavus* cultures grown for 7 days in a medium containing starch, triglycerides, and zein. Concentrations are expressed in mg/mL of culture medium ($n = 3$). Error bars are not shown for arabitol, erythritol, and trehalose data for clarity of presentation.

followed by a substantial decrease during the remainder of the fermentation period (**Figure 5**). Mannitol was produced in greater concentrations than any of the other fungus-specific materials. Kojic acid concentrations rose after 2 days to a peak (1.5 mg/mL) at day 6.

DISCUSSION

The medium utilized in this study presented *A. flavus* with the major corn storage materials simultaneously. Hydrolysis of both starch and triglycerides occurred in a nonselective manner after 18 h of incubation. This effect is evident from the relatively rapid breakdown of starch accompanied by a significant increase in reducing sugar concentrations (**Figure 2**), as well as a large increase in free fatty acid concentrations during the same period (**Figure 4**). This interesting phenomenon contrasts sharply with observations made with *A. flavus* in a medium simulating cottonseed. In that case, the fungus selectively utilizes raffinose, the low molecular weight storage trisaccharide from cotton (*4*). Thus, *A. flavus*, when faced with utilization of three complex carbon sources, metabolized two of them (starch and triglycerides) simultaneously. As hydrolysis of starch produced increasing glucose concentrations, triglyceride hydrolysis tapered off. However, triglyceride hydrolysis increased again, evidenced by increasing fatty acid concentrations from day 6 to day 7 (**Figures 3** and **4**), as glucose concentrations dropped below 2 mg/mL. Although there was little statistical difference between fatty acid concentrations during days 5−7 of the fermentation period, data trends suggest that free fatty acid concentrations were increasing. Sufficient triglyceride remained to provide substrate for hydrolysis. Zein concentrations did not decrease significantly until after day 3 of the fermentation period (**Figure 2**), demonstrating a fungal preference for starch or triglycerides as a carbon source. The peak of zein concentration noted at 36 h was probably caused by difficulty in removing all of the corn lipids from the hydrophobic storage protein.

The maximum rate of aflatoxin B_1 production coincided with the maximum rate of biomass production (**Figure 1**). Biomass production continued to increase after 4 days, albeit at a lower

rate, whereas aflatoxin levels decreased from the peak at 4 days. The final pH of the fungal medium was also coupled to aflatoxin production (**Figure 1**). Medium pH dropped rapidly in synchrony with aflatoxin B_1 production and began to increase after the peak of aflatoxin production. This phenomenon has been observed before in *A. flavus* (*10*) and *Aspergillus parasiticus* (*11*) in defined media, as well as in media simulating cottonseed (*4*). The complete significance of this pH/aflatoxin production relationship is not fully understood, but pH regulatory elements may be associated with the aflatoxin biosynthetic gene cluster (*12*). In addition, the fungus may be altering the pH of its microenvironment to maximize the effectiveness of secreted hydrolytic enzymes.

The principal saccharide released from starch hydrolysis was glucose, as expected. *A. flavus* is known to produce amylase, glucoamylase, and other hydrolases that contribute to starch digestion (*13*). Glucose is central in primary metabolic pathways and is required for cell wall biosynthesis. The observed release of glucose via starch hydrolysis apparently influenced both *A. flavus* metabolism and breakdown of complex carbon sources. Once glucose concentrations reached sufficient levels, catabolic repression of hydrolase production was observed. Much lower concentrations of larger glucose oligomers (dimer, trimer, etc.) were detected; all displayed peak concentrations on day 2 of the fermentation period (**Figure 3**). Hydrolysis of oligomers apparently rapidly continued to completion to produce monomers. The data reinforce the prominence of glucose in the metabolic priorities of *A. flavus*.

Concentration profiles of released fatty acids closely paralleled each other (**Figure 4**), indicating that no fatty acid was preferentially utilized by the fungus. This same phenomenon was observed with *A. flavus* fermentations using media simulating cottonseed (*4*).

Concentration profiles of most fungus-specific metabolites were similar to those observed in cottonseed simulation media (*4*). Mannitol was produced in the highest concentrations and peaked at day 4 (**Figure 5**). Maximum mannitol production coincided with maximum aflatoxin B_1 and biomass production. Mannitol may be involved in osmoregulation (*14*) and serve as a protectant from both oxidative and osmotic stress (*15*). This substance would appear to provide a critical metabolic function in the growth of *A. flavus*. Arabitol, erythritol, and trehalose, thought to serve as fungal storage metabolites (*14*), were detected in much lower concentrations than mannitol (**Figure 5**).

Alhtough the corn simulation medium more closely approximates in planta conditions than normally used defined fungal media, certain aspects of the model system differ greatly from mature seed. When the fungus is infecting corn kernel tissues, it must surmount barriers such as a waxy layer and seed coat. Even after the seed coat has been breached, the primary substrate targets are not readily accessible to the fungus. Storage proteins and triglycerides are sequestered in dedicated storage bodies (lipid bodies and protein bodies). Thus, the fungus must hydrolyze membrane barriers before these potential substrates are available. None of these barriers are encountered by the fungus in this model system. In addition, starch exists as tightly packed granules in the endosperm tissue of a corn kernel. During heat sterilization of the corn simulation medium, the structure of these starch granules is gelatinized. This process effectively hydrates the starch molecules and presumably allows for more rapid hydrolysis by fungal enzymes. Hydrolysis of starch reserves in planta would probably proceed at slower rates than in the simulation medium, unless ample water was present in the endosperm.

Kojic acid concentration profiles indicated peak production occurred late in the fermentation period (**Figure 5**). This is one characteristic commonly attributed to secondary metabolites. This observation contrasts with aflatoxin, a compound frequently called a secondary metabolite that formed primarily during the rapid growth phase. Concepts linking secondary metabolism to growth phase may have limited utility in media with varying compositions. Production of kojic acid in the cottonseed simulation media was not observed. Kojic acid is the fungal metabolite responsible for bright green-yellow fluorescence (BGYF) material that is used as a diagnostic indicator of *Aspergillus* presence in some commodities (*16*). It is interesting to note that kojic acid was produced in corn simulation media, and BGYF is often observed on infected corn kernels in the field. Similarly, the absence of kojic acid production in cottonseed simulation media is accompanied by a lack of BGYF in infected cottonseed kernels, although it does occur on cotton lint. By this measure, these defined medium systems incorporating seed reserve materials represent good models for their respective commodities and may serve as useful tools for the investigation of divergent regulation of aflatoxin biosynthesis in different crops.

ACKNOWLEDGMENT

The technical assistance of A. Klaman, D. Downey, and S. Pelitire is greatly appreciated.

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Received for review August 7, 2001. Revised manuscript received October 23, 2001. Accepted October 29, 2001.

JF011048E